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Decreased expression of Kv7 channels in Hirchsprung's disease

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ABSTRACT

Purpose: Voltage-dependent K^+ channels (Kv channels) participate in electrical rhythmicity and smooth muscle responses and are regulated by excitatory and inhibitory neurotransmitters. Kv channels also participate in the interstitial cell of Cajal (ICC) and smooth muscle cell (SMC) responses to neural inputs. The Kv family consists of 12 subfamilies, Kv1-Kv12, with five members of the Kv7 family identified to date: Kv7.1-Kv7.5. A recent study identified the potassium channel Kv7.5 as having a role in the excitability of ICC-IM in the mouse colon. We therefore designed this study to test the hypothesis that Kv7 channels are present in the normal human colon and are reduced in Hirschprung's disease (HSCR).
Material and methods: HSCR tissue specimens were collected at the time of pull-through surgery (n = 10), while normal control tissue specimens were obtained at the time of colostomy closure in patients with imperforate anus (n = 10). Kv7.3-Kv7.5 immunohistochemistry was performed and visualized using confocal microscopy to assess their distribution. Western blot analysis was undertaken to determine Kv7.3-Kv7.5 protein quantification.
Results: Kv7.3 and Kv7.4-immunoreactivity was co-localized with neuron and ICC markers, while Kv7.5 was found to be expressed on both ICCs and SMCs. Western blot analysis revealed similar levels of Kv7.3 and Kv7.5 expression in the normal colon and HSCR colon, while Kv7.4 proteins were found to be markedly decreased in ganglionic specimens and decreased further in aganglionic specimens.
Conclusion: A deficiency of Kv7.4 channels in the ganglionic and aganglionic bowel may place a role in colonic dysmotility in HSCR.

Ion channels are essential for basic cellular function and for processes including sensory perception and intercellular communication. Voltage-dependent K^+ channels (Kv channels) participate in electrical rhythmicity and smooth muscle responses by regulating the resting membrane potential in various cell types throughout the body [1]. As they are regulated by excitatory and inhibitory neurotransmitters, Kv channels also participate in the ICC and SMC responses to neural inputs. The Kv family of K^+ channels consists of 12 subfamilies, Kv1–Kv12, within which five members of the Kv7 family have been identified. These comprise Kv7.1–Kv7.5, and are encoded by the genes KCNQ1–5. To date, most published data regarding the expression of these channels have focused on their location in the heart and brain [2].

The enteric nervous system (ENS) is an intricate network of neurons and glia that lies within the walls of the gastrointestinal tract. It is the largest and the most complex division of the peripheral nervous system, containing more neurons than the spinal cord, and has the unique characteristic that it can carry out its motility functions completely independent of the central nervous system. Hirschsprung's disease (HSCR) is a congenital condition characterized by an absence of enteric ganglia in the distal colon. This condition is caused by a failure of neural crest cell migration to the distal hindgut during the early stages of embryonic development, which results in a tonically contracted aganglionic segment [3]. Surgical resection of this aganglionic segment of colon is the only treatment available to HSCR patients. A recent study identified the potassium channel, Kv7.5, as having a role in the excitability of ICC-IM in the mouse colon [4]. We therefore designed this study to test the hypothesis that Kv7 channels are present in the normal human colon and are reduced in Hirschsprung's disease.

1. Materials and methods

1.1. Tissue samples

This study was approved by the Ethics Medical Research Committee, Our Lady's Children's Hospital, Dublin, Ireland (Ref. GEN/292/12) and tissue samples were obtained with informed parental consent. HSCR specimens from 10 patients who underwent pull-through surgery were studied. These specimens were divided into aganglionic and ganglionic samples. HD patients were aged 6 ± 3 months old. We compared the most distal aganglionic segments with the most proximal ganglionic segments. Normal control colon samples included 10 specimens from patients who

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underwent colostomy closure for imperforate anus. Control samples were taken from patients who were 11 ± 4 months old. Tissue specimens were either snap-frozen in liquid nitrogen and stored at −80 °C for protein extraction or embedded in OCT Mounting Compound (VWR International, Leuven, Belgium) for immunofluorescence and stored at −80 °C until use.

1.2. Immunofluorescence staining and confocal microscopy

Frozen blocks of HSCR colon and normal control samples were sectioned transversely at a thickness of 10 μm, mounted on SuperFrost® Plus slides (VWR International, Leuven, Belgium) and fixed with 10% buffered formalin for 5 min. Sections underwent cell membrane permeabilization with 1% TritonX-100 for 20 min at room temperature. After blocking with 10% normal goat serum (Sigma Aldrich Ltd., Arklow, Ireland) for 30 min to avoid non-specific absorption, sections were incubated with primary antibodies; rabbit anti-Kv7.3, mouse anti-Kv7.4 and rabbit anti-Kv7.5 (Abcam, Cambridge, UK), mouse anti-HuC/HuD (Molecular Probes), mouse anti-α-smooth muscle actin (Dako, Ireland), rabbit anti-protein gene product 5.5 (Sigma Aldrich, Ireland), mouse anti-c-kit and rabbit anti-c-kit (Abcam, Cambridge, UK) and rabbit anti-TMEM (Santa Cruz), all used at dilution 1:100, overnight at 4 °C. Sections were then washed in PBS + 0.05% Tween and incubated with corresponding secondary antibodies (goat anti-rabbit Alexa Fluor® 488, dilution 1:200 and goat anti-mouse Alexa Fluor® 647, dilution 1:200, Abcam, Cambridge, UK) for 1 h at room temperature. After washing, sections were counterstained with DAPI antibody, dilution 1:1000 (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min, washed, mounted and coverslipped with Fluorescent Mounting Medium (DAKO Ltd., Cambridgeshire, UK). All sections were independently evaluated by two investigators with a LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

1.3. Protein extraction and western blot

Specimens of HSCR colon and control colon were homogenized in RIPA buffer (Radio Immunoprecipitation Assay, Sigma-Aldrich Ltd., Wicklow, Ireland) containing 1% protease inhibitor cocktail (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland). Protein concentrations were determined using a Bradford assay (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland). A total volume of 20 μl Laemmli sample buffer (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland) containing 10 μg of protein was loaded in the 10% SDS-PAGE gel (NuPAGE Novex Bis–Tris gels, Invitrogen, Carlsbad, USA) for electrophoretic separation. The electrophoresis was performed in MES SDS running buffer (Invitrogen, Carlsbad, USA). Proteins were transferred to a nitrocellulose membrane (Millipore Corporation, Billerica, USA) by western blotting. Membranes were then blocked in 3% BSA – 0.05% Tween for 30 min prior to antibody detection. Primary antibodies against rabbit anti-Kv7.3, mouse anti-Kv7.4 and rabbit anti-Kv7.5 (Abcam, Cambridge, UK), dilution 1:1000, were used and incubation was performed overnight at 4oC. Following further washes, the membranes were incubated with the appropriate secondary antibodies (goat anti-rabbit IgG, HRP-linked Antibody, dilution 1:10,000, and goat anti-mouse IgG-HRP, dilution 1:10,000, Abcam, Cambridge, UK) respectively followed by washing. Detection was performed with the ECL plus chemiluminescence kit (Thermo, Fisher Scientific, Dublin, Ireland). We used GAPDH (mouse anti-GAPDH, dilution 1:1000, Abcam, Cambridge, UK) as an additional loading control. The Mann–Whitney test was used to determine statistical significance as

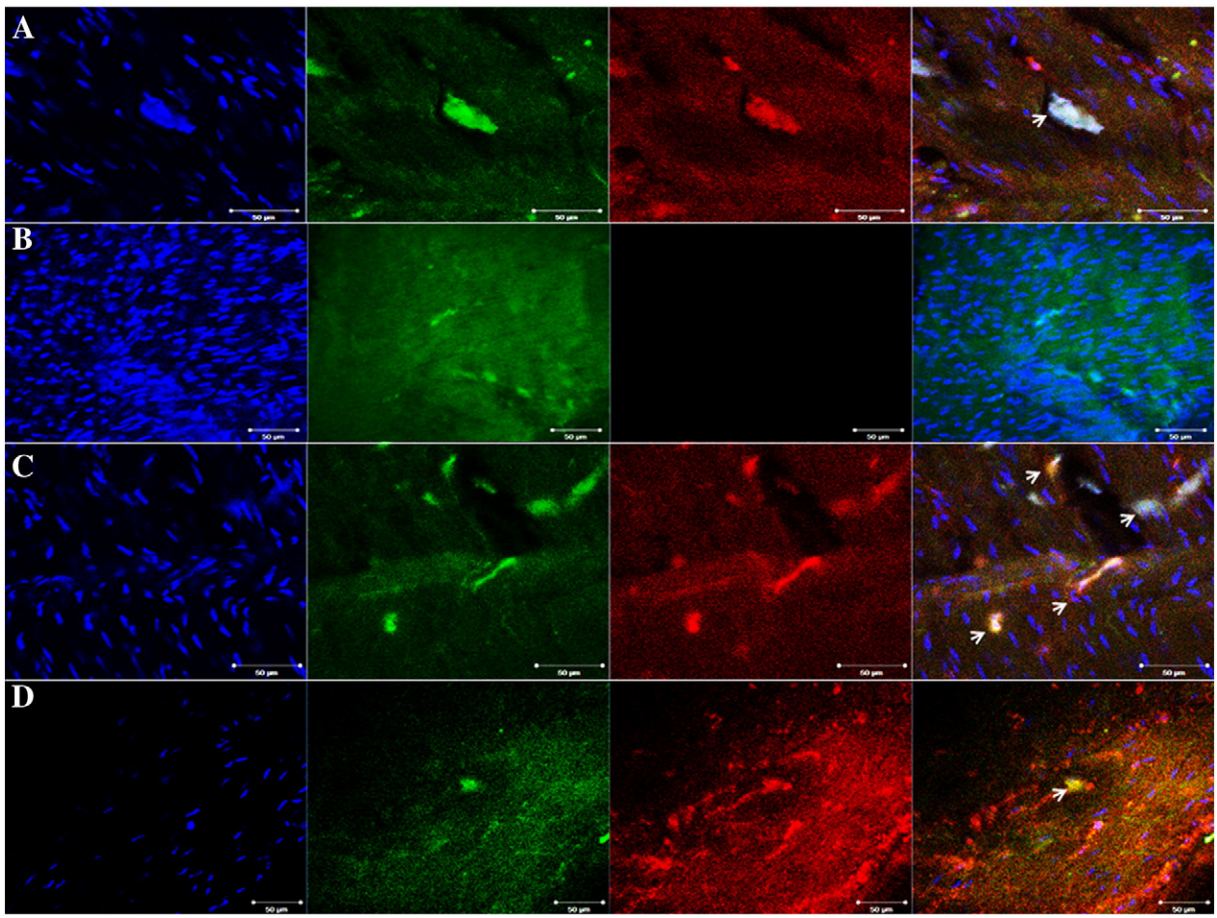

Fig. 1. Immunofluorescent staining of Kv7.4 channels (green) co-localized with HuC/D (red) labeled neurons in ganglionic (A) and absent in the aganglionic specimens (B). Kv7.4 was also co-localized with c-kit (red) labeled ICCs in ganglionic (C) and aganglionic specimens (D). Nuclei were stained with DAPI (blue). Arrows show co-localisation.